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Methylation of *Herpesvirus saimiri* DNA in lymphoid tumor cell lines

(intracellular virus DNA/restriction endonucleases/lymphoma/virus-producing and nonproducing cells)

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ABSTRACT **Several continuous lymphoid cell lines have been established from tumors induced by *Herpesvirus saimiri*. At least a portion of the viral DNA in the marmoset lymphoid cell line 1670, which does not produce detectable virus, is present as covalently closed circular episomal DNA. The use of restriction endonuclease digestion, transfer to nitrocellulose filters, and hybridization of the virus-specific DNA has produced strong evidence that viral DNA sequences present in total 1670 cell DNA and in isolated episomes are extensively methylated. The restriction endonuclease *Hpa* II has the same recognition sequence as *Msp* I but, unlike *Msp* I, fails to cleave when the C of the C-G dinucleotide is methylated. Viral DNA sequences of 1670 cells are refractory to cleavage by *Hpa* II but not *Msp* I; greater than 80% of the *Hpa* II cleavage sites appear to be methylated. Similarly, viral DNA sequences of 1670 cells are refractory to cleavage by *Sma* I (C-C-C-G-G-G) and *Sac* II (C-C-G-C-G-G) but not *Sac* I, *Pvu* II, or *Pst* I, which lack the dinucleotide C-G in their recognition sequences. Methylation of mammalian DNA has been previously found exclusively at C residues in the dinucleotide C-G. *H. saimiri* DNA sequences of another nonproducer cell line, 70N2, also appeared to be extensively methylated, but analysis of total cell DNA extracted from three virus-producing lymphoid lines revealed no evidence of methylation of viral DNA sequences. It remains to be seen if methylation of viral DNA plays a role in the lack of complete expression of *H. saimiri* genome information in nonproducing lymphoid cell lines.**

Herpesvirus saimiri is an indigenous virus of squirrel monkeys (*Saimiri sciureus*) that causes no overt disease in its natural host. Most adult squirrel monkeys have a latent *H. saimiri* infection that persists for life. The same virus causes lymphoma and death in various other New World primates (1). The virus is similar in some respects to Epstein–Barr virus. Epstein–Barr virus is a suspected causative agent of Burkitt lymphoma, a human tumor prevalent in regions of central Africa and New Guinea (2).

The virion DNA of *H. saimiri* is unusual in structure, containing a marked intramolecular heterogeneity (3). The unusual features of the genomic DNA separate *H. saimiri* from the herpes simplex virus group and the cytomegalovirus group. Most virion genomes consist of what has been called M-DNA. M-DNA molecules of *H. saimiri* consist of one long L-DNA region (71×10^6 daltons) of low density (36% G+C) and two terminal H-DNA regions of variable length and high density (71% G+C) (3). The H-DNA regions at both ends are composed of as many as 30 identical repeat units of 830,000 daltons oriented in the same direction. M-DNA is infectious. Small amounts (5–10%) of very long defective H-genomes are also present in virus preparations.

At least 10 continuous lymphoid cell lines have been established from *H. saimiri*-induced tumors. The cell line 1670, obtained originally from a *H. saimiri*-induced marmoset lymphoma (4), has been the most extensively studied. Although no detectable virus is produced by this cell line, approximately 0.4% of the total 1670 cell DNA is *H. saimiri* DNA (5). As in most lines carrying Epstein–Barr virus, at least a portion of the viral DNA is present as covalently closed circular episomal DNA (6). It is not known for *H. saimiri*-transformed lymphoid lines whether a portion of the viral information is integrated with host cell DNA. The results of partial denaturation mapping with the electron microscope indicate an unusual arrangement of sequences in the episomal DNA (6).

We have begun a restriction enzyme analysis of episomal DNA from 1670 cells, using the transfer technique of Southern (7) in order to map the viral DNA sequences. In the course of these analyses we observed unusual properties of the H-DNA sequences with some restriction enzymes but not with others. The results reported here provide strong evidence for the methylation of *H. saimiri* DNA in nonproducing lymphoid lines.

Cellular DNAs of mammals contain 2–6% of their cytosine residues as 5-methylcytosine (m^5C), and this accounts for virtually all of the methylated bases in mammalian DNA (8–10). No generally accepted function has been ascribed to this methylation. The dinucleotide GG is the least common of the 16 possible dinucleotides, and m^5C has been found exclusively in this dinucleotide (10). The virion DNA molecules of adenovirus and herpes simplex virus, however, contain at most 1/50th as much m^5C (as % of total cytosine) as cellular DNA does (9, 11).

MATERIALS AND METHODS

Materials. The sources for restriction endonucleases were as follows: *Bam*HI, *Msp* I, *Pst* I, *Pvu* II, and *Sac* I from New England BioLabs; *Hpa* II from Miles; *Sma* I and *Sac* II, unpublished procedures. Cesium chloride (Suprapur) was from EM Laboratories (Elmsford, NY). Agarose (low electroendosmosis) was from Sigma. [6-^{3}H]Uridine (NET-156) and [α-^{32}P]dNTPs (300–600 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were obtained from New England Nuclear. Cell culture material was from GIBCO, nitrocellulose filters (BA85) from Schleicher & Schuell, and *Escherichia coli* DNA polymerase I and calf thymus DNA from Boehringer Mannheim.

Cells and Virus. *H. saimiri* no. 11 was grown in owl monkey kidney cells (OMK) and purified as described (3). The lymphoid cell lines 1670 and 70N2 were originally established from

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Abbreviations: NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate; NaDodSO$_4$, sodium dodecyl sulfate; *Sma* I, restriction endonuclease from *Serratia marcescens* (other restriction endonucleases are abbreviated similarly).

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marmosets bearing *H. saimiri*-induced tumors (4, 12); these cell lines no longer produce virus. The cell lines L77/5 and 1926 were established from the tumorous lymph nodes of marmosets injected with *H. saimiri* (5, 12); both of these cell lines were producing virus during the course of this work. The lymphoid line H1591 was recently established by transformation *in vitro* of cotton-topped marmoset lymphocytes with the owl monkey strain of *H. saimiri*.§ All lymphoid cell lines were grown in stationary suspension cultures in RPMI 1640 medium containing 10% heat inactivated fetal calf serum. Periodic attempts were made to rescue *H. saimiri* from cells of each lymphoblastoid culture by cocultivation with permissive OMK cells.

Isolation of DNA. DNA was prepared from each cell line essentially as described (6). Briefly, Sarkosyl-lysed cells were incubated overnight with 0.5 mg of proteinase K per ml (not preincubated) and DNA was isolated from CsCl equilibrium centrifugation in a Spinco vertical 50 VTi rotor. DNA was dialyzed, precipitated with ethanol, and gently dissolved in 10 mM Tris·HCl, pH 7.5. After the UV spectrum was recorded, EDTA was added to give 1 mM, and the DNA was stored at 2°C. Virion DNA was prepared as described (3).

Preparation of *H. saimiri* DNA ^{32}P-Labeled *In Vitro*. The procedure used for "nick repair" of *H. saimiri* no. 11 DNA was based on that described by Mackey *et al.* (13). [α-^{32}P]dNTP (500 μCi) was taken to near dryness by evaporation at room temperature. The following were then added to produce the indicated concentration in a final volume of 100 μl: *H. saimiri* DNA (33 μg/ml), the remaining three nonradioactive dNTPs (100 μM each), Tris·HCl at pH 7.5 (0.01 M), KCl (0.05 M), $MgCl_2$ (0.05 M), and *E. coli* DNA polymerase I (300 units/ml). Each batch of DNA polymerase or DNA was pretested at micro scale for rate of incorporation before use as above. After incorporation at 15°C was maximal, the reaction was stopped by addition of 60 μl of 0.1 M EDTA at pH 7.5 and incubated at 65°C for 7 min. The mixture was then chromatographed on a Sephadex G-50 column at 4°C in 10 mM Tris·HCl, pH 8.0/20 mM NaCl/2 mM EDTA. The void volume fractions containing [^{32}P]DNA were pooled and stored at −20°C. Specific activities of greater than 10^8 cpm per μg (Cerenkov) were always obtained.

Restriction Endonuclease Digestion of DNA, Agarose Gel Electrophoresis, Transfer of DNA to Nitrocellulose Filters, and Hybridization with *H. saimiri* [^{32}P]DNA. DNA was digested with *Sma* I either in 6 mM each of Tris·HCl at pH 7.5, NaCl, $MgCl_2$, and 2-mercaptoethanol at 36°C or in 10 mM Tris·HCl at pH 9.0, 5 mM $MgCl_2$, and 15 mM KCl at 30°C. Digestion with *Sac* II was performed in 6 mM each of Tris·HCl at pH 7.5, $MgCl_2$, and 2-mercaptoethanol and 20 mM NaCl at 36°C. Digestion with other enzymes was carried out according to the recommendation of the supplier. Agarose electrophoresis was performed in a semihorizontal slab gel apparatus with 25 × 16 cm gel dimensions at 50 V at room temperature. The electrophoresis buffer (0.089 M Tris/0.089 M boric acid/2.5 mM Na_2EDTA, final pH 8.3) was recirculated during electrophoresis. DNA in gels was stained with ethidium bromide at 1 μg/ml in electrophoresis buffer, the stained DNA was photographed under UV light, and the DNA was transferred from gels onto nitrocellulose filters by the procedure of Southern (7). *H. saimiri* [^{32}P]DNA to be used for hybridization was denatured in the same tube with 100 μg of calf thymus DNA in 0.25 M NaOH at 67°C as described (5), neutralized, and diluted with 3× standard saline citrate (NaCl/Cit; 0.15 M NaCl/0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (NaDodSO_4). After the nitrocellulose filters containing transferred DNA had been rinsed and baked, the filters were saturated with 3× NaCl/Cit and 0.1% $NaDodSO_4$, rolled end to end, and inserted into a test tube containing the denatured *H. saimiri* [^{32}P]DNA and calf thymus DNA. The 3× NaCl/Cit and 0.1% $NaDodSO_4$ buffer was added to completely cover the top of the filter, the solution was mixed well, and hybridization proceeded at 67°C for 20 hr. The filter was blotted dry and rinsed in 4 liters of 2× NaCl/Cit and 0.1% $NaDodSO_4$ containing 6.9 g of NaH_2PO_4, 13.4 g of $Na_2HPO_4 \cdot 7H_2O$, and 2.8 g of $Na_4P_2O_7 \cdot 10H_2O$ at 67°C for 3 hr. The filter was then air dried, placed on Kodak X-Omat R film, and incubated in sealed plastic at −70°C for autoradiography using Du Pont Lightning-plus screens.

Quantitation of Cytosine Methylation. Each lymphoid cell line, diluted 1:3, and pre-confluent OMK cells were labeled with [6-^{3}H]uridine at 1 μCi/ml for 3 days. This procedure labels cytosine, thymine, and m^{5}C in DNA (ref. 9; unpublished). DNA was isolated as described above (yielding 35,000–100,000 cpm per μg of DNA) and hydrolyzed with 88% (wt/vol) formic acid at 150°C for 30 min (9, 14). The released purine and pyrimidine bases along with standards were separated by descending paper chromatography in 2-propanol/concentrated HCl/H_2O (680:176:144, vol/vol). This solvent system adequately separates adenine, guanine, uracil, cytosine, m^{5}C, and thymine. The UV spots containing cytosine and m^{5}C were cut out and soaked overnight in water. An aliquot of each was analyzed for radioactivity content and another aliquot was rechromatographed in the above solvent to precisely quantitate the radioactivity in cytosine and m^{5}C. The ratio of cpm in cytosine to cpm in thymine in each cell line varied slightly but was very close to 1. The radioactivity in cytosine and thymine accounted for greater than 90% of the total.

RESULTS

Comparison of *H. saimiri* DNA Cleavage with *Hpa* II and *Msp* I. *Hpa* II has the same recognition sequence as *Msp* I (C-C-G-G), but, unlike *Msp* I, it fails to cleave when the C of the C-G dinucleotide is methylated (15). Fig. 1 *left* (slots C and D) shows that no. 11 virion DNA was cleaved identically by *Msp* I and *Hpa* II in the L- and H-DNA regions. The supermolar bands in the 100,000- to 170,000-dalton range are derived from cleavage in the repetitive H-DNA sequences. The analysis of viral DNA in 1670 cells with these enzymes is shown in Fig. 1 *right*. Line 1670 cell DNA cleaved with *Msp* I produced strongly hybridizing *H. saimiri* H-DNA sequences in the 100,000- to 170,000-dalton region (Fig. 1 *right*, slot D). After cleavage of 1670 cell DNA with *Hpa* II, however, most of the *H. saimiri* H-DNA sequences migrated much more slowly in the 0.7% agarose gel (Fig. 1 *right*, slot B). The supermolar H-sequences of 1670 DNA were clearly refractory to cleavage by *Hpa* II (slot B) but not *Msp* I (slot D). The *H. saimiri* H-DNA fragments produced by *Hpa* II cleavage of 1670 cell DNA indicated only about 1 in 10 recognition sites were actually cleaved. That the very broad smear obtained with *Hpa* II-digested 1670 DNA in Fig. 1 *right* (slot B) was indeed derived from H-DNA sequences has been verified by hybridization to H-[^{32}P]DNA specific probe (data not shown). Although it is not shown clearly in Fig. 1 *right*, L-DNA of 1670 cells is also resistant to cleavage by restriction endonucleases inhibited by a methylated C-G dinucleotide.

Cleavage of *H. saimiri* DNA with Other Restriction Enzymes. Originally we set out to map episomal viral DNA from 1670 cells by these techniques, using enzymes for which maps of virion no. 11 DNA are available. This approach depended heavily on the use of *Sma* I because this enzyme conveniently cleaves four times in each repeat unit of H-DNA but does not

§ B. Fleckenstein, I. Müller, C. Muldes, and M. D. Daniel, unpublished data.

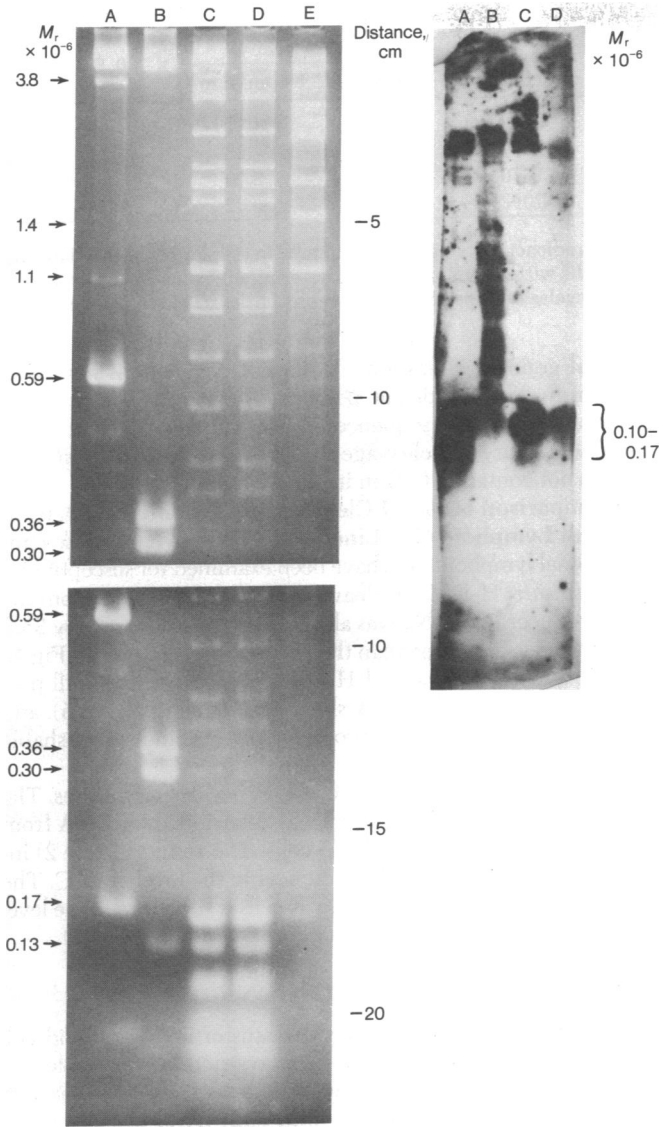

FIG. 1. Digestion of *H. saimiri* DNA with *Msp* I and *Hpa* II. (*Left*) A 0.8-μg sample of virion DNA (no. 11) was digested with the indicated enzyme and subjected to electrophoresis through a 1.5% agarose gel. The DNA bands were stained by soaking the gel 1 hr in ethidium bromide at 1 μg/ml in electrophoresis buffer. The gel was placed on an UV light box and the fluorescent pattern was photographed. The gel was photographed in two sections because the UV light box area was not large enough for the entire gel. The two pictures are thus from the same gel. No. 11 DNA was digested with: slot A, *Sac* II; B, *Sma* I; C, *Msp* I; D, *Hpa* II. Slot E contained adenovirus type 2 DNA fragments (*Bam*HI; *Eco*RI) as molecular weight standards. (*Right*) Total 1670 cell DNA (3.5 μg) or no. 11 virion DNA (6.9 ng) was digested with quantities of the indicated enzymes more than sufficient for complete digestion and subjected to electrophoresis for 18 hr through a 0.7% agarose gel. The DNA in the gel was transferred to a nitrocellulose filter by the technique of Southern (7), and the DNA on the filter was hybridized with no. 11 [^{32}P]DNA. The supermolar products in the 100,000- to 170,000-dalton range derived from virion H-DNA are indicated. Slot A, no. 11 and *Hpa* II; B, 1670 and *Hpa* II; C, no. 11 and *Msp* I; D, 1670 and *Msp* I.

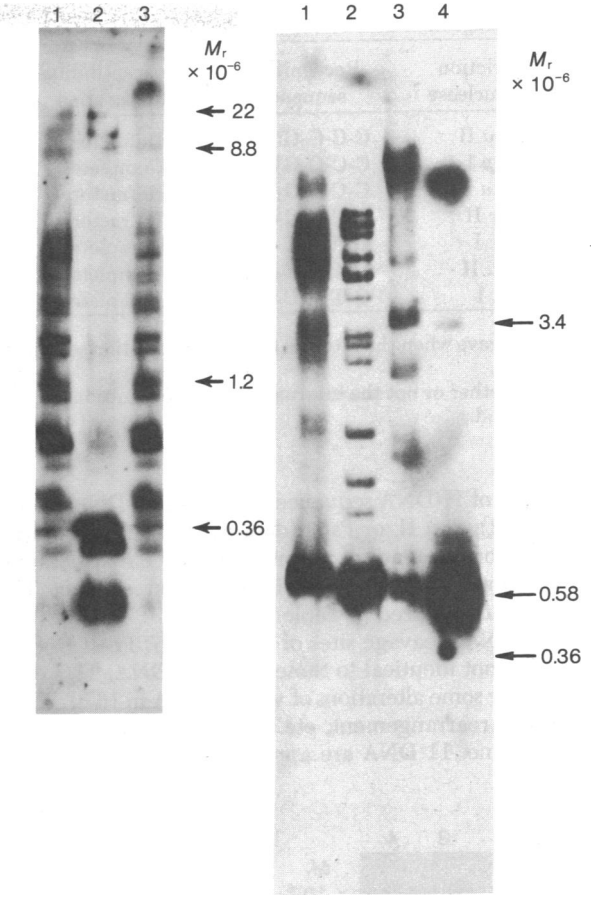

FIG. 2. *H. saimiri* H-DNA of 1670 cells is refractory to cleavage by *Sma* I and *Sac* II but not *Sac* I. DNA was digested, transferred to nitrocellulose filters, and hybridized as described in the legend of Fig. 1 *right*. (*Left*) Slot 1, 1670 and *Bam*HI + *Sma* I; 2, no. 11 and *Bam*HI + *Sma* I; 3, 1670 and *Sma* I. (*Right*) Slot 1, 1670 and *Sac* 1; 2, no. 11 and *Sac* I; 3, 1670 and *Sac* II; 4, no. 11 and *Sac* II.

cleave L-DNA. H-Sequences of total 1670 cell DNA (Fig. 2 *left*) and of isolated episomes (data not shown) were also resistant to cleavage by *Sma* I (recognition sequence C-C-C-G-G-G). The unusual behavior of virus-specific DNA in 1670 cells with some restriction enzymes was first observed with *Sma* I and has been seen with all preparations of 1670 cell DNA tested (more than five) under a variety of conditions. Methylation of C of the C-G dinucleotide apparently blocks the action of *Sma* I (16). The restriction endonuclease *Bam*HI (G-G-A-T-C-C) cleaved four times in virion L-DNA but did not cleave in virion H-DNA. As expected, *Bam*HI cleaved L-DNA of the 1670 cell DNA but did not alter the *Sma* I-resistant H-DNA fragments (Fig. 2 *left*, slot 1).

Most trivial explanations of these observations have been eliminated by the following experiments. Total 1670 cell DNA prepared with and without a phenol and chloroform isolation step produced the same *Sma* I resistance pattern. Use of 5 times more *Sma* I than shown in Fig. 2 *left* (more than 10 times that needed for complete digestion) did not alter the resistance pattern. It is unlikely that there was anything present in the 1670 cell DNA preparation (EDTA, proteinase K, etc.) that was inhibiting *Sma* I; when total 1670 cell DNA was mixed with no. 11 virion DNA, the virion DNA was cleaved normally (data not shown).

Similarly, H-DNA sequences of total 1670 cell DNA were refractory to cleavage by *Sac* II (C-C-G-C-C-G-G) (Fig. 2 *right*). *Sac* II cleavage of virion M-DNA produces 67,000,000- and 3,800,000-dalton fragments from the L region and 590,000- and 165,000-dalton supermolar fragments from the H region. It is our contention, based on band intensities in Fig. 2 *right*, column 3, that the numerous fragments between 590,000 and 8,000,000 daltons in the *Sac* II digest of 1670 DNA result from resistance of the H-DNA to digestion by *Sac* II.

Table 1. Susceptibility of H-DNA sequences in total 1670 cell DNA to digestion with various restriction endonucleases

Restriction endonuclease	Recognition sequence	Susceptibility to digestion
Hpa II	C-C-G-G*	Refractory
Msp I	C-C-G-G†	Complete
Sma I	C-C-C-G-G-G	Refractory
Sac II	C-C-G-C-G-G	Refractory
Sac I	G-A-G-C-T-C	Complete
Pvu II	C-A-G-C-T-G	Complete
Pst I	C-T-G-C-A-G	Complete

* Does not cleave when the internal C of the recognition sequence is methylated.

† Cleaves whether or not the internal C of the recognition sequence is methylated.

Cleavage of H-DNA sequences of 1670 Cell DNA by *Sac* I (Fig. 2 *right*), *Pvu* II, or *Pst* I (data not shown) was indistinguishable from cleavage of virion DNA. *Sac* I, *Pvu* II, and *Pst* I lack the dinucleotide C-G in their recognition sequence. These results are summarized in Table 1.

The L-DNA cleavage sites of *Sac* I in 1670 cell DNA are similar but not identical to those in virion DNA. This can be explained by some alterations of viral L-DNA in 1670 cells due to deletion, rearrangement, etc. Although many similarities with virion no. 11 DNA are apparent, it is also obvious that several genome alterations in the L-DNA regions have also occurred. The important point for this study is that *Sac* I cleavage of H-DNA sequences in 1670 cell DNA appears to be identical with *Sac* I cleavage of H-DNA from virions and *Sac* I does not contain a C-G in its recognition sequence.

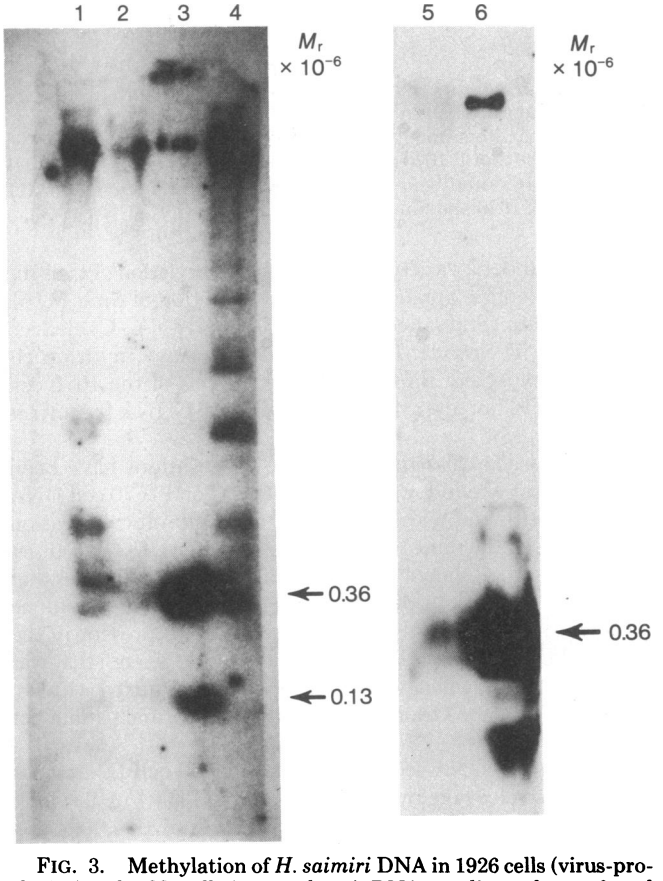

FIG. 3. Methylation of *H. saimiri* DNA in 1926 cells (virus-producing) and 70N2 cells (nonproducer). DNA was digested, transferred to nitrocellulose filters, and hybridized as described in the legend of Fig. 1 *right* except a 1.0% agarose gel was used. Slot 1, 70N2 and *Sma* I; 2, 1926 and *Sma* I; 3, no. 11 and *Sma* I; 4, 1670 and *Sma* I; 5, 1591 and *Sma* I; 6, no. 11 and *Sma* I.

Table 2. Percent of total cytosine present as 5-methylcytosine*

Cell line	$\frac{\text{cpm in } m^5C}{\text{cpm in C + cpm in } m^5C} \times 100$, %
OMK	4.77
L77/5	4.23
1670	3.70
70N2	3.72

* The radioactivity in cytosine and m^5C in cellular DNA after labeling of cells with [6-^{3}H]uridine was determined by acid hydrolysis and two cycles of paper chromatography as described in the text.

Comparison of *Sma* I Cleavage of *H. saimiri* DNA from Several Lymphoid Cell Lines. Total cell DNAs from several additional lymphoid lines have been examined for susceptibility of *H. saimiri* H-DNA to cleavage by *Sma* I. H-DNA from the nonproducer line 70N2 was also refractory to cleavage by *Sma* I but to a lesser degree than the nonproducer line 1670 (Fig. 3, slots 1 and 4). Cleavage of H-DNA from three lines still producing virus—1926 (Fig. 3, slot 2), H1591 (Fig. 3, slot 5), and L77/5 (data not shown)—produced patterns indistinguishable from no. 11 virion H-DNA.

Determination of Level of m^5C in Several Cell Lines. The degree of cytosine methylation was determined for DNA from OMK, 1670, 70N2, and L77/5 cells. The results (Table 2) indicate at most only small differences in the level of m^5C. The level of m^5C determined for OMK cells is very close to the level previously reported for human embryo kidney cells (9).

DISCUSSION

Viral H-DNA in five *H. saimiri*-transformed lymphoid cell lines has been analyzed with seven restriction endonucleases; three of the cell lines were virus producers and two were not. The results strongly indicate that viral DNA in cells of the nonproducing lines 1670 and 70N2 contains cytosine methylations absent from virion DNA and from viral DNA extracted from lymphoid cell lines that produce virus. It should be noted, however, that these techniques give only indirect evidence for DNA methylation. Unfortunately, the level of viral DNA in these lymphoid cell lines is too small to readily measure the degree of methylation by presently available techniques. Modifications of DNA other than methylation have not been described in eukaryotes.

There is precedence for this restriction enzyme resistance–methylation analysis. Many restriction enzymes with recognition sites containing C-G do not cleave ribosomal DNA of higher eukaryotes properly because of the presence of a 5-methyl group at the internal C residue of these sites (17, 18). Calf satellite DNA has a similar resistance to digestion with some restriction endonucleases that has been ascribed to methylation (16). It is interesting to note that integrated adenovirus DNA in some tranformed cell lines resists digestion by some restriction endonucleases and this may also be due to methylation (19).

1670 and 70N2 cells appear to differ quantitatively in the degree to which viral H-DNA sequences are methylated. There appears to be no correlation of the degree of total cell DNA methylation and the degree of methylation of viral DNA in the individual cell lines.

H. saimiri resembles Epstein–Barr Virus in that neither virus particles nor structural antigens can be detected in tumor cells. When tumor tissue is freshly explanted into culture, however, virus is produced at least for a period of time (usually several months) before the cells generally revert to a nonproducing status (20, 21). In this report we have correlated the presence of *H. saimiri* DNA methylation with tumor cell lines not producing virus. *H. saimiri* DNA in tumor cell lines still producing virus is apparently not methylated appreciably.

The significance of viral DNA methylation in nonproducing tumor cell lines is not clear at this time. Methylation of DNA has been associated with the decreased expression of hemoglobin genes in a mouse cell system (22). Mechanisms in which methylation is involved in viral persistence without productive infection can be envisioned. It is conceivable that DNA methylation may be a contributing factor in determining permissive vs. nonpermissive infection, virus latency, and in some cases tumorigenesis. Further work will be needed to determine if DNA methylation plays a role in or is only trivially associated with the suppression of complete virus expression.

It will be interesting to see if viral DNA in *H. saimiri*-induced tumors and viral DNA from cells transformed by the human lymphotropic herpesvirus, Epstein–Barr Virus, are similarly methylated.

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